Could fluorinated pharmaceuticals have an impact on the EOF amount in human blood?

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Bachelor thesis (30 hp)
Örebro University, 2022

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Abstract

Recent studies have discovered an increasing amount of unknown organofluorine in human blood. This increase insinuate that humans might be exposed to potentially new and unidentified organofluorine compounds. Currently there is a large gap between the amount of per- and polyfluorinated substances (PFAS) being monitored and the number of PFAS existing. Due to regulations of legacy PFAS like perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS), production have shifted to new PFAS, mainly short-chains (C4-C6). New approaches have been made in order to discover these emerging PFAS, for example total oxidizable precursor (TOP) assay and extractable organofluorine (EOF) analysis. Though, these methods are non-specific and therefore useful in screening of fluorinated compounds. In today's pharmaceutical market, a lot of drugs contain fluorine, and there is a current increase in number of fluorinated pharmaceuticals. Since EOF analysis measures all organofluorine that can be extracted, the presence of fluorinated pharmaceuticals in a sample may contribute to the EOF amount. However, if fluorinated pharmaceuticals do co-extract during sample preparation for common PFAS analysis is not yet known. This study evaluates if selected pharmaceuticals (fluoxetine, seproxetine, celecoxib and ciprofloxacin) will be co-extracted during ion pair extraction, a common extraction for EOF analysis. The extraction was first optimized in order to gain a good extraction efficiency for the pharmaceuticals. Ion pair solution adjusted to different pH's (pH 3-4, pH 10 and pH 12) was tested, celecoxib had a good recovery independent of what pH was used (95%-102%), fluoxetine and seproxetine were only extractable at high pH's (10 and 12) and had a recovery of around 90%, ciprofloxacin, however, could not be extracted no matter a low or high pH. The extraction was then conducted both with a pH of 10 and with a low pH (3-4) on human sera samples (n=6); the sera samples included 3 samples where subjects had been taking fluoxetine and 3 control samples. When analysis of the extracted sera samples was performed, no detectable level of pharmaceutical including its known metabolite seproxetine was found. An EOF and TF analysis of the sera samples were also performed. The EOF analysis could however not indicate any presence of pharmaceutical due to contamination in the procedural blanks. The TF analysis indicated vaguely a higher amount of total fluorine in the sera samples where subjects had taken fluoxetine compared to the control group; however, the large variation between the two groups make it hard to draw any conclusion. Since fluoxetine, seproxetine and celecoxib could be extracted when spike tests were performed, hypothetically they could contribute to the EOF amount. However, since no detectable level was found in the sera samples after extraction it cannot be stated for sure that these pharmaceuticals can be extracted from human blood, using ion-pair extraction. The unanswered question here is if the pharmaceutical is present in some other form in the sera samples, which cannot be extracted, or if it is even present at all.

1. Introduction

Per- and polyfluorinated substances (PFAS) are a large group of mostly synthetic chemicals which have been produced since the 1950's. (Kissa, 2001). PFAS have been released to the environment through industrial manufacturing and through consumer use of PFAS containing products (EPA, 2017), such as makeup, food packaging materials and clothing (Buck et al., 2011, Prevedouros et al., 2006, Schultes et al., 2018). By definition, any chemical with at least a perfluorinated methyl group (−CF3) or a perfluorinated methylene group (−CF2−) without any H/Cl/Br/I atom attached to the group is a PFAS (OECD, 2021). Some of the compounds containing a perfluoroalkyl moiety have unique properties which makes them prone to bioaccumulation and long-range environmental transport (Miralles-Marco and Harrad, 2015). Due to the widespread use of PFAS, many substances have been detected in the environment, wildlife, and humans (Yamashita et al., 2005, Higgins and Luthy, 2006, Jahnke et al., 2007, Hansen et al., 2001, Taniyasu et al., 2005). The concerns emerged in the early 2000's when the hazards and widespread occurrence of one of today's most studied PFAS (perfluorooctane sulfonic acid; PFOS) was reported in wildlife around the whole world (Giesy and Kannan, 2001). Perfluorooctanoic acid (PFOA) together with PFOS are the two most commonly detected PFAS and have been found in both human blood and wildlife samples (Giesy and Kannan, 2001, Hansen et al., 2001). There have been negative health effects observed from PFOA and PFOS which have resulted in regulations to reduce and limit the release of PFAS (Stockholm Convention, 2022, Sharkey et al., 2020) Studies have shown that occupational exposure to PFOA can be associated with prostate cancer, cerebrovascular disease, diabetes (Lundin et al., 2009) and ulcerative colitis.(Steenland et al., 2013) Both PFOS and PFOA have been linked to changes in birthweight and low sperm quality among the general human population (Apelberg et al., 2007; Joensen et al., 2009). Another study found results that indicated immunotoxicity linked to prenatal exposure to PFOA in the general population (Granum et al., 2013). PFOS and PFOA have also been associated with late puberty amongst children living near a Chemical Plant (Lopez-Espinosa et al., 2011).

PFOS were included in the Stockholm convention in 2009 and PFOA in 2019 and the main producer, 3M, phased out production of both compounds in 2002 in the United States (EPA, 2017, Stockholm Convention, 2022). Following this, production has shifted to other PFAS like short-chain PFAS (C4-C6) (Wang et al., 2017, Wang et al., 2013) which have been considered to have lower impact on health and environment because of lower tendency to bioaccumulate (Martin et al., 2003).

Analysis of both legacy PFAS (long chain PFAS phased out of production) and novel PFAS (emerging PFAS and polyfluorinated compounds used as replacements for legacy PFAS) is not an easy task. Due to PFAS having a diversity of chemical properties, it is difficult to develop a sufficient method that eliminates matrix enhancement or suppression of lipophilic components in the sample. Different approaches have been used to overcome this and to improve the quantification results, for example isotope dilution and matrix matched calibration curves. Despite this, problems remain, since currently not all isotope labeled reference standards or matrices representative for all samples are available. The current most promising method to overcome matrix effects seems to be to reduce the initial sample volume and the amount of extract injected (Nakayama et al., 2019). However, there is a gap between the commonly measured PFAS and the PFAS humans are exposed to (Aro et al., 2021). Different approaches have been made in order to try to fill this gap (Rotander et al., 2015, Houtz and Sedlak, 2012, Miyake et al., 2007). Identification using non-target methods has become the main approach in discovering as-yet-unknown PFAS (Nakayama et al., 2019).

Analysis of organofluorine (OF) has been found to be a useful non-specific method for analyzing PFAS. One method is to measure the extractable organofluorine (EOF) analysis using combustion ion chromatography (Miaz et al., 2020). Since all PFAS contain fluorine, the EOF amount will measure even the yet to be identified PFAS. In addition to PFAS, the EOF could also contain other organofluorine compounds, and recent organofluorine studies have shown an increase of unidentified organofluorine (UOF) in humans while the concentration of legacy PFAS, for example PFOS has been decreasing (Yeung et al., 2015, Miaz et al., 2020). This increase suggests that humans are being exposed to new and unidentified fluorinated products (Yeung et al., 2015). The increasing number of fluorinated pharmaceuticals could be contributing to the increasing trend of UOF (Inoue et al., 2020, Aro et al., 2021). However, if these pharmaceuticals in fact do have an impact on the EOF amount is not known since it is not yet verified if fluorinated pharmaceuticals are co-extracted with the PFAS during sample preparation (Aro et al., 2022). The overall goal of this study is to evaluate if fluorinated pharmaceuticals may contribute to EOF in human blood. This was done by evaluating if selected fluorinated pharmaceuticals (seproxetine, fluoxetine, celecoxib and ciprofloxacin) might be co-extracted with a common PFAS extraction method called ion pair extraction. Other objectives of this study were; 1) to study the differences of total fluorine in subjects taking fluorinated pharmaceuticals compared to subjects that are not, and 2) to modify the extraction method by adjusting the pH to improve the extraction efficiency of selected fluorinated pharmaceuticals.

2. Background

2.1 Definition and properties of PFAS.

PFAS are divided into two subgroups: perfluorinated substances and polyfluorinated substances. Perfluorinated substances are compounds where all the hydrogens on all carbons, except any carbon associated with a functional group, have been replaced by fluorines. Polyfluorinated substances are however compounds were only some (not all) hydrogens have been replaced by fluorine atoms (EPA, 2017). The definition of PFAS have varied through the years, the newest revised definition was made in 2021 by the Organization for Economic Cooperation and Development (OECD). The definition of PFAS according to OECD are stated as: “PFASs are defined as fluorinated substances that contain at least one fully fluorinated methyl or methylene carbon atom (without any H/Cl/Br/I atom attached to it), i.e., with a few noted exceptions, any chemical with at least a perfluorinated methyl group (−CF3) or a perfluorinated methylene group (−CF2−) is a PFAS”. The exceptions refer to a carbon atom with a H/Cl/Br/I atom attached to it. (OECD, 2021).

Due to the high electronegativity and small atomic size of the fluorine atoms, the PFAS structure gives them properties like high surface activity, chemical and thermal stability, and water and oil-repellency (Kissa, 2001). This chemical and thermal stability of the perfluorinated methyl or methylene groups and their lipophilic and hydrophilic nature, in addition to the extremely strong and stable C-F bond, makes PFAS properties very useful. Although PFAS can be used by themselves, they can also be used in production of polymers and surfactants (Kissa, 2001). Polymers have been used in textile impregnation and greaseproof papers and surfactants takes advantage of the surface tension-reduction properties. Some applications are coatings and aqueous film–forming foams (AFFFs) used to extinguish fires involving highly flammable liquids (Buck et al., 2011).

Short and long chained PFAS can be distinguished based on the length of the fluorinated carbon chain. Perfluorocarboxylic acids (PFCAs) with a fluorinated carbon chain with eight carbons or more and perfluorosulfonic acids (PFSAs) with a carbon chain with six carbons or more are referred to as long chain PFAS. The length of the carbon chain can affect the physicochemical properties of the

compound which will influence their behavior in the environment and in organisms as well as their bioaccumulation factor and toxicity (OECD, n.d.). The longer chained PFAS are of higher concern since they are more prone to bioaccumulation and PFOA and PFOS are the two long-chained PFAS most often reported and discussed in scientific literature (Buck et al., 2011). Although short-chained PFAS may be less bioaccumulative, they can be more mobile (Chambers et al., 2021, Brendel et al., 2018) and recent studies have also shown similar toxicities to longer chain analogues (Palazzolo et al., 2022, Pierozan et al., 2022).

2.2 Regulation and trend for new PFAS

Some regulations and control measures have been made for PFAS other than the two most common studied PFAS i.e., PFOA and PFOS. As stated in the introduction, it is common to replace long-chained PFAS to shorter chained ones (Wang et al., 2013). However, there are still a lot of PFAS that are being overlooked, even some that have structural similarities to PFOA and PFOS or their precursors, that are still being produced (Wang et al., 2013, Wang et al., 2014, Wang et al., 2015). The common practice of replacing long-chained PFAS with shorter-chained and the current neglect of a great amount of PFAS is still of huge concern. Whang et al. (2017) presented three reasons why; 1) the assessment and potential management actions for these additional PFAS will need large additional time, resources research and regulatory efforts, 2) It is technically and financially difficult to identify and reverse human and environmental exposure to PFAS, and 3) the increasing number of PFAS will prolong and increase the previous two challenges just mentioned.

Recent research has discovered “new” PFAS in the environment, (Nakayama et al., 2019). A non-target analysis of PFAS in pooled fish samples from China discovered over 300 novel PFAS. (Liu et al., 2018). Due to the shift in manufacturing alternatives and replacements for some PFAS, recent research has been focusing on detecting and identifying new PFAS. However, developing methods that can cover a broad range of PFAS have been proven hard. The recent approach has been to use non-target methods to identify many new PFAS. Still, the lack of standardized sample pretreatment methods and data analysis procedures makes this approach only suitable for discovery rather than comprehensive analysis. Non-specific approaches are useful for screening fluorinated substances, one disadvantage however is that the results cannot be used to evaluate toxicological effects (Nakayama et al., 2019). As mentioned before, there have been different approaches in trying to develop methods for discovering new PFAS. Some of these are total oxidizable precursor (TOP) assay (Houtz and Sedlak, 2012) and extractable organofluorine (EOF) analysis (Miyake et al., 2007). Although new approaches to discovering novel PFAS has been done there is still a long way to go. Currently there is very little data of the emerging PFAS, for example potential human health outcomes due to exposure of these compounds. A lot of research is needed in order to understand and characterize their behavior in the environment and to understand potential human health risks. Even though research progress has been made for novel PFAS, there is likely still hundreds to thousands of unknown PFAS yet to be characterized. Therefore, development of non-target methods to determine unknown PFAS is of high importance (Brase et al., 2021).

2.3 Extractable organofluorine analysis

A common non-specific method is measuring extractable organofluorine (EOF) by combustion ion chromatography (CIC). This method was first presented by Miyake et al., in 2007 and has since then been used for analyses of water, sediment, biological samples (Miyake et al., 2007), and to detect PFAS in cosmetics (Schultes et al., 2018). To measure the amount of extractable organofluorine (EOF), organic compounds are first extracted prior to the analysis to improve the selectivity (Nakayama et al., 2019). The extracts are then combusted at a high temperature to measure the

amount of released fluorine. This method often uses combustion ion chromatography (CIC) where it is possible to measure the levels of OF compounds without knowing the structure and properties of the compounds (Miaz et al., 2020). To understand how much of the EOF is contributed by known PFAS, the extracted sample is divided where one part undergoes targeted analysis, and one part undergoes EOF analysis (Yeung et al., 2009). Since it is a non-target analysis it will measure all OF compounds, including the unidentified PFAS and other OF compounds. The CIC cannot distinguish between organic and inorganic fluorine which also have been found in human blood, therefore it is important to separate these during sample preparation (Miaz et al., 2020). The amount of EOF can be compared with the amount of fluorine that would likely be present from the target PFAS, the difference is then believed to come from unidentified organofluorine (UOF) compounds. Studies have identified unknown extractable organofluorine in different matrices like fresh water, aquatic invertebrates, rats (Koch et al., 2019, Yeung et al., 2009), and also an increase of UOF in humans has been observed (Yeung et al., 2015, Miaz et al., 2020). The increase in humans have been suggested to be due to the increasing number of fluorinated pharmaceuticals (Aro et al., 2021).

2.4 Fluorinated pharmaceuticals

Fluorinated pharmaceuticals first arrived in the 1950's and today around 20% of the commercial pharmaceuticals are so-called fluoro-pharmaceuticals (Inoue et al., 2020). However, this percentage is increasing, between 2001-2011 around 40 new pharmaceuticals containing fluor were introduced (Zhou et al., 2016). Selective fluorination has become a traditional approach when creating new drugs to increase the pharmaceutical effectiveness, biological half-life and binding affinity (Böhm et al., 2004, Kirk, 2006, Hagmann, 2008, Shah and Westwell, 2007). These benefits have resulted in that some of the best-selling and best-performing drugs in today's pharmaceutical market being fluorinated pharmaceuticals (Zhou et al., 2016), such as Prozac (fluoxetine) (Rossi et al., 2004).

2.4.1 Fluoxetine & Seproxetine

Fluoxetine (trade name: Prozac, figure 3) is a fluorinated pharmaceutical used in treatment of depression, OCD, and bulimia (Rossi et al., 2004). it works as a selective inhibitor of serotonin re-uptake, with little effect on other neurotransmitters (Wong et al., 1995). Since its release in 1988 it has become the most widely prescribed antidepressant in the world (Rossi et al., 2004). Fluoxetine is absorbed well from the gastrointestinal tract (Fulton and McTavish, 1995) after oral intake, with peak plasma concentrations after 6-8 hours. It has a half-life of about 2-3 days, however its active metabolite norfluoxetine (seproxetine) has a half-life of 7-9 days. Fluoxetine is 94% bound to protein and becomes metabolized in the liver (Guze, 1994). The dosage of the drug is usually 20 mg/day for adults, but it can range from 5-80 mg/day. It is also common that the dosage might increase over time during treatment (Guze, 1994). Fluoxetine contains equal amount of R- and S-enantiomers, resulting in both R- and S-norfluoxetine (Wong et al., 1995).

Seproxetine is the S-enantiomer of fluxoetine's active metabolite called norfluoxetine (figure 5) (PubChem, 2022). and is formed by demethylation of the parent compound (Wong et al., 1995). Both fluoxetine and seproxetine are amines that contain a fully fluorinated carbon atom (PubChem, 2022). It works in the same way as fluoxetine, i.e., a selective inhibitor of serotonin re-uptake, but seproxetine has never been marketed alone as a drug. The S-enantiomer is more potent than the R-enantiomer in the inhibition of serotonin re-uptake (Wong et al., 1995).

A study measured the plasma concentration of fluoxetine and seproxetine after 8 weeks of taking the drug. The patients were administered 20 mg/day of fluoxetine and the concentrations were determined with gas chromatography. The mean value of fluoxetine and seproxetine in all patients were 97 ng/mL

and 128 ng/mL respectively, this resulted in a mean sum value of 225 ng/mL (Amsterdam et al., 1997).

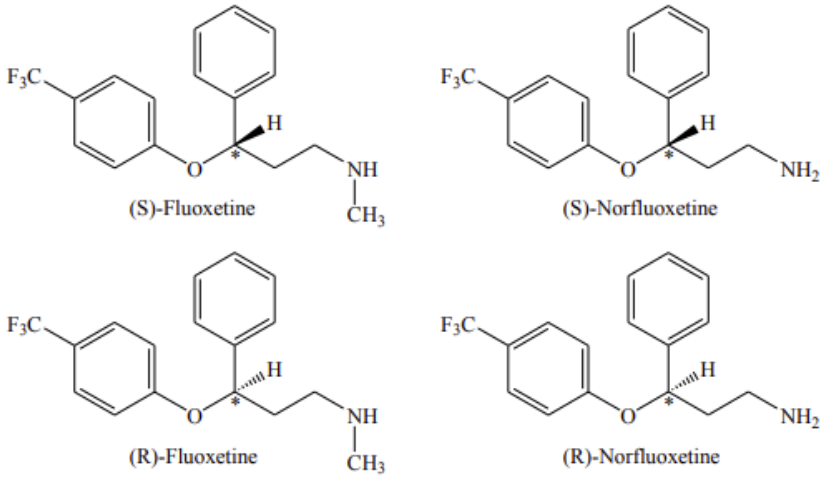


Figure 3. Chemical structures of (S)-(R) fluoxetine and (S)-(R) norfluoxetine.
(S)-Norfluoxetine = Seproxetine (Carvalho et al., 2017).

2.4.2 Celecoxib

Celecoxib (trade name: Celebrex; figure 4) is the 21st most sold fluorine-containing drug in the U.S market and is a nonsteroidal anti-inflammatory drug (Zhou et al., 2016). The compound is a sulfonamide and a member of both toulenes and pyrazoles and it contains a fully fluorinated carbon atom (PubChem, 2022). It is used to reduce pain in various conditions, for example arthritis and related disorders. The mechanism of celecoxib is to block prostaglandin synthesis by selective inhibition of cyclo-oxygenase-2 (COX-2) (Zhou et al., 2016). After oral intake, celecoxib is rapidly absorbed to the gastrointestinal tract and peak serum and plasma concentrations are shown after approximately 3 hours (Gong et al., 2012), The drug is extensively protein bound (>97%), primarily to albumin (Davies et al., 2000), and is metabolized extensively in the liver, only 3% leaves the body unchanged. It is primarily metabolized to hydroxycelecoxib through methyl hydroxylation but can be further oxidized to carboxycelecoxib. However, none of the metabolites are active (Gong et al., 2012). The common doses administered are 100 mg two times a day or 200 mg once a day, and the half-life is around 11 hours (Zeng et al., 2015, Davies et al., 2000). Following a single oral dose of 200 mg given to healthy test subjects (n = 36, 19-52 years), the peak plasma level was reached after 3 hours with a C_{max} of 705 ng/mL. With multiple dosing, steady-state concentration is reached on day 5 or before. (FDA Approved Drug Products: CELEBREX (celecoxib) oral capsules)

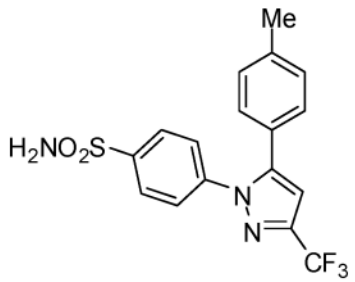


Figure 4. Chemical structure of celecoxib (Zhou et al., 2016).

2.4.3 Ciprofloxacin

Ciprofloxacin is a second-generation fluoroquinolone (Zhang et al., 2018) and is used to treat various bacterial infections, for example certain urinary tract infections, lower respiratory infections, and skin infections. It contains one flour atom as well as a carboxyl group and is also an aminoquinoline (figure 5) (PubChem, 2022). Ciprofloxacin is the active substance in many antibiotics and works against many gram-positive and gram-negative bacteria. Its mechanism of action is to inhibit the enzymes called topoisomerase II (DNA gyrase) and topoisomerase IV. This kills the bacteria since the enzymes are needed for bacterial DNA replication, transcription, repair, and recombination. Ciprofloxacin is generally administered in 250, 500 or 750 mg every 12 hours over a period of time which is 7-14 days but can be shorter and longer. The half-life in serum is around 4 hours in subjects with normal renal function. (FDA Approved Drug Products: Cipro).

A study administered the drug orally every 12 hours over a 7-day period at doses of 250, 500 and 750 mg to healthy volunteers. Peak concentration in serum was achieved after 1-1.5 h. Mean peak serum levels were 1.35-1.42 µg/mL after the 250 mg dose, 2.60-2.89 µg/mL after the 500 mg dose and 3.41-4.21 µg/mL after the 750 mg dose.

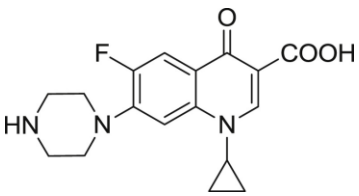


Figure 5. Chemical structure of Ciprofloxacin (Zhang et al., 2018)

3. Method & materials

3.1 Chemicals

The methanol (MeOH) used was of HPLC grade (>99.8%) and LC grade (>99.9%) from Fisher Scientific (Trinidad and Tobago) The Milli-Q water (18.2 MΩ) was from the Örebro University's Millipore system. Tert-butyl methyl ether (MTBE) of HPLC grade (>99,8%) was obtained from Sigma-Aldrich (France). Tetrabutylammonium bisulfate (TBA, 99.0%) were from Sigma Aldrich (USA). The ammonium hydroxide (25%) was obtained from Fisher Scientific (UK), the silver nitrate ($AgNO_3$) (>99.0%) was from Sigma-Aldrich (USA). Acetonitrile of HPLC grade (≥99.9 %) was from Fisher Scientific (China). 2-propanol of LC/MS grade was obtained from Fisher Scientific (The Netherlands), and ammonium acetate (NH_4Ac, eluent additive for LC/MS) was obtained from Sigma-Aldrich (USA).

The standard of native PFAS (PFCAs, C4-C14, C16, C18; PFSAs C4-C10, C12; perfluorooctanesulfonamide; fluorotelomer sulfonic acids, 4:2, 6:2, 8:2) and mass-labeled PFAS (PFCAs, C4-C12; PFSAs, C2, C4, C8; perfluorooctanesulfonamide 6:2 and 8:2 fluorotelomer sulfonic acids.) where obtained from Wellington laboratories (Guelph, Ontario, Canada). For more information about each standard mixture (CS, IS and RS) can be found in the Appendix, Tables 8-10.

3.2 Samples

Horse serum was used as sample matrix during the method optimization of the ion pair extraction. The horse serum was from Håtunalab AB, the company follows the ISO 9001 standard for quality.

Further, a standard reference material (SRM) freeze-dried human serum (SRM 1957) was used for method evaluation. The SRM human sera was from National Institute of Standards and Technology (NIST) and named Organic Contaminants in Non-Fortified Human Serum.

A total of six blood samples (sera) from the U.S. were analyzed for fluorinated pharmaceuticals. An EOF and a TF analysis were also conducted on the same samples. The samples were provided by Prof. Thomas Webster from the Boston University School of Public Health, and they were purchased commercially. The six samples included three control samples where the subjects had not taken any pharmaceutical and three sera samples where the subjects had taken the pharmaceutical fluoxetine. As the human sera samples in this project were commercial products which were not associated with any personal information, ethical permit is not required.

3.3 Pharmaceuticals

Stock solutions for the pharmaceuticals were obtained and used to prepare 2 µg/mL standard solutions. The stock solutions of the pharmaceuticals were the following: for fluoxetine; Fluoxetine hydrochloride - Supelco Cerilliant certified reference material (1.0 mg/mL as free base in MeOH), for seproxetine; Norfluoxetine oxalate - Supelco Cerilliant certified reference material (1.0 mg/mL as free base in MeOH), for celecoxib; Celecoxib – Sigma-Aldrich, Pharmaceutical secondary standard, certified reference material (salt dissolved to 1.0 mg/mL in methanol), and for ciprofloxacin; Ciprofloxacin – Fluka ≥98% (HPLC) (salt dissolved to 1.0 mg/mL in methanol).

3.4 Extraction

The extraction method used was ion pair extraction (IPE) based on the described method by Hansen et al. (2001). In short, the extraction tests were conducted using both MilliQ water and horse sera as sample matrix. Procedural blanks, recovery samples and afterspike samples were done in duplicates where one duplicate was prepared with 0.5 mL of MilliQ water and the other with 0.5 mL horse serum. The recovery samples were then spiked with an internal standard (IS) mixture of PFAS and the pharmaceuticals (40 ng of fluoxetine, seproxetine and celecoxib, 80 ng of ciprofloxacin) before extraction for target analysis. A recovery standard (RS) mixture of PFAS were spiked after the extraction to the recovery samples. The procedural blanks, recovery samples and afterspike samples then followed the same extraction procedure. For the afterspike samples, IS, RS and the pharmaceuticals were spiked after the extraction.

In short, 2 mL of 0.5 M tetrabutylammonium (TBA) was added to the 0.5 mL of sample matrix, 5 mL of MTBE was then added, and the samples were vortexed. The samples were then shaken horizontally for 15 minutes and centrifuged at 8000 g for 10 minutes. The organic phase (upper layer) was then collected and transferred to a new tube. The extraction was repeated twice with 3 mL of MTBE and the extracts were combined and evaporated gently down to 0.2 mL with nitrogen gas. They were then reconstituted to 1.0 mL with methanol and evaporated further to 0.5 mL before they were transferred to LC vials, the PP tubes were washed 3 times and the vials were evaporated down to a final volume of 0.5 mL and spiked with RS befor analysis. The extract was split into three aliquots, one part were transferred to a new LC vial for target analysis, one part was put in CIC vial for EOF analysis and the extract left in the original vial was used to analyze the pharmaceuticals.

For the target analysis, IS and RS were added to the procedural blanks. The 100 µL (+ IS and RS) of extract was then evaporated down to 40 µL. 60 µL of aqueous mobile phase was added to the extract to obtain a 40% organic solvent content for target PFAS analysis.

The extraction efficiency is evaluated by calculating the extraction recovery for the pharmaceuticals. This is done by dividing the area of the pharmaceuticals in the samples with the area of the pharmaceuticals in the afterspike samples. The use of the afterspike samples rather than the solvent standard was done in order to leave out the possible ion suppression/enhancement.

3.4.1 Evaluation of the ion pair extraction method at different pH's

In order to evaluate the extraction efficiency of the four different pharmaceuticals several spike tests with different pH's were conducted. A spike test using the ion-pair extraction was done without any pH adjustment, which results in a pH of around 3-4 since this is the pH of the TBA solution. An alkaline version of the ion-pair extraction was tested using both pH 10 and 12. The pH was adjusted by adding 25% ammonia hydroxide to the TBA solution.

3.5 Extraction of sera samples

The serum samples had already been analyzed before where ion pair extraction without any pH adjustment was used (Aro et al., 2021). In present study, the same sample was extracted again with an alkaline ion-pair extraction (a pH of around 10). The extraction procedure was done in the exact same way as described above (3.3). MilliQ procedural blanks as well as SRM procedural blanks both without any pH adjustment and with a pH of around 10 were used in the extraction in order to check for contamination. A spike recovery sample with the four pharmaceuticals (40 ng, 80 ng ciprofloxacin) was also included, as well as an afterspike sample in order to check matrix effects. All samples were split for analysis of the pharmaceuticals and EOF analysis.

3.5.1 Total fluorine analysis of sera samples

A total fluorine (TF) analysis of the sera samples was also performed where 100 µL of pure sera sample were diluted with 100 µL of MilliQ water. To avoid high chloride content when analyzing the samples an amount of approximately 0.05 g of $AgNO_3$ were added to the samples, the samples were then centrifuged at 5500 g for 10 minutes to form a pellet. The supernatants were transferred to CIC vials for direct TF analysis.

3.6 Instrumental analysis

3.6.1 Pharmaceuticals

The pharmaceuticals were measured using a supercritical fluid chromatography (SFC) system (Waters Ultra Performance Convergence Chromatograph, UPCC; Waters Corporation, Milford) coupled to a Xevo TQ-S micro (Waters Corporation) tandem mass spectrometer (MS/MS), with a Torus DIOL analytical column (3.0 × 100 mm, 1.7 µm). The mobile phases that were used were CO_2 and MeOH with 0.05% ammonia. In the instumental development stage, Torus 2-PIC (3.0 × 100 mm, 130Å, 1.7 µm), HSS C18 SB (3.0 × 100 mm, 1.8 µm) and acquity UPCC BEH (3.0 × 100 mm, 1.7 µm) columns were evaluated, as well as different compositions of mobile phase in order to improve the chromatography of ciprofloxacin. The different mobile phases tested were MeOH with 0.5% ammonia, MeOH with 0.1% formic acid and 100% MeOH.

3.6.2 Target PFAS

A total of 25 target PFAS were measured using a Waters ultra-performance liquid chromatograph (UPLC) coupled to a triple quadrupole mass spectrometer (Xevo TQS-MS/MS). The mobile phases used were 2 mmol/L ammonium acetate in 70:30 milliQ:MeOH (mobile phase A) and 2 mmol/L ammonium acetate in 100% MeOH (mobile phase B). The ionization mode used was negative

electrospray ionization (ESI) and the analysis was carried out in multiple reaction monitor mode (MRM). The parameters for each compound can be found in table 1 in the appendix.

3.6.3 EOF & TF

The EOF and TF levels were measured using a CIC system with a combustion module (Analytik Jena, Germany), a 920 Absorber module (Metrohm, Switzerland) and a 930 Compact IC Flex ion chromatograph module (Metrohm, Switzerland). To separate the anions an ion-exchange column (Metrosep A Supp 5-150/4.0) was used. An isocratic elution was used at a flow rate of 0.7 mL/min. The mobile phase used was a carbonate buffer composed of 64 mmol/L sodium carbonate and 20 mmol/L sodium bicarbonate and the absorber solution was milliQ water. The EOF and TF concentrations were calculated by using an external calibration curve made with PFOA standards (50−1000 ng/mL F).

3.7 Quality assurance & Quality control measures

Prior to the extraction tests with the pharmaceuticals, an extraction with the ion pair method was done with native PFAS in order to check the recovery and repeatability. The results indicated recoveries in a range of 50 to 130% for target PFAS and a good repeatability (RSD 8-25%)

Every batch of samples included at least three procedural water blanks and at least three procedural blanks with the matrices that were used (horse sera or SRM). These were used for target analysis of PFAS in order to keep track of any PFAS contamination and also to check EOF contamination. When analyzing the blood samples a spike recovery sample and an afterspike sample was included using SRM, this was done to check the recovery of pharmaceuticals in order to check the extraction efficiency on human sera.

For the EOF and TF analysis, several combustion blanks was conducted to reach a stable fluoride signal. Combustion blanks were also used between samples in order to avoid possible carryover between samples. Repeated injections of standard solution with a known EOF amount to monitor the performance of the system (RSD = 6%). Before analysis a five-point external calibration curve was made, using 50, 100, 250, 500, 1000 and 2000 ng F/mL solutions. This resulted in a R2= 0.999 linearity. The limit of detection (LOD) was determined as the lowest point of the calibration curve, 50 ng F/mL for EOF. Since the samples were diluted by 1:1 for the TF analysis, the LOD was 100 ng/mL.

The instrumental LOD values for the pharmaceuticals were determined as the lowest point of a calibration curve. The LOD for seproxetine, fluoxetine and celecoxib were 0.5 ng/mL while ciprofloxacin had a LOD of 60 ng/mL.

The LOD values for the target PFAS were determined by taking the average level of each PFAS plus 3 times the standard deviation of the procedure blanks. If the procedure blank did not contain any analyte, the LOD was determined by using the lowest point of the calibration curve. Due to some PFAS contamination problems, the LOD was calculated for each batch of the extraction tests. The LOD values can be found in table 7 in the appendix.

4. Results & Discussion

4.1 Instrumental development of selected fluorinated pharmaceuticals

The instrumental method for analyzing the selected fluorinated pharmaceuticals on the UPCC (Waters Ultra Performance Convergence Chromatograph, UPCC; Waters Corporation, Milford) were developed and optimized based on different compositions of the mobile phase as well as different columns. The different columns and mobile phase compositions (see section 3.5.1) were mainly tested in order to improve the chromatographic separation for ciprofloxacin, however it resulted in only minor differences. The chromatograms for ciprofloxacin for the four different columns tested (Torus DIOL, BEH, Torus 2-PIC, and HSS C18 SB) can be seen in the appendix on pages 29-30. When testing the different mobile phase composition, the mobile phase with 0.5% NH_4OH (with Torus DIOL column) resulted in some improvement, the chromatogram can be seen in the appendix on page 30. However, the mobile phase and column was chosen after what gave the best chromatography for fluoxetine and seproxetine since these pharmaceuticals was of highest interest considering the blood samples. The initial column and mobile phase composition tested resulted in a good chromatography for fluoxetine, seproxetine and celecoxib and where therefore chosen (Torus DIOL column with 0.05% NH_4OH in MeOH as mobile phase). The chromatogram for fluoxetine, seproxetine, celecoxib and ciprofloxacin using the Torus DIOL column with 0.05% NH_4OH in MeOH as mobile phase can be seen in the appendix on pages 30-32.

4.2 Effect of pH on extraction efficiency of fluoxetine, seproxetine and celecoxib

The ion pair extraction method was used due to its low contamination of OF, which makes it suitable for EOF analysis. This method does not co-extract inorganic fluoride which is important since CIC cannot distinguish between inorganic fluoride and organofluorine (Hansen et al., 2001).

4.2.1 Ion-pair extraction without pH adjustment (pH 3-4)

The extraction efficiency and matrix effect of ion-pair extraction, when no pH adjustment was done can be seen in figure 6, where the error bars are the standard deviation of the replicates. The ion pair extraction where the pH was not adjusted (i.e., around 3-4) resulted in around 100% recovery for celecoxib in the MilliQ water samples and in the horse sera samples. Fluoxetine and seproxetine had very poor recovery at approximately 5% when the pH was not adjusted. There was ionization suppression present for all the pharmaceuticals and in both milliQ water samples and horse sera samples, this is however compensated for in the graphs. The ionization suppression effect is presented in table 1. For celecoxib it was around 25-28%, for seproxetine and fluoxetine it was considerably lower (1-5%), however this is due to the recovery being almost non-existent.

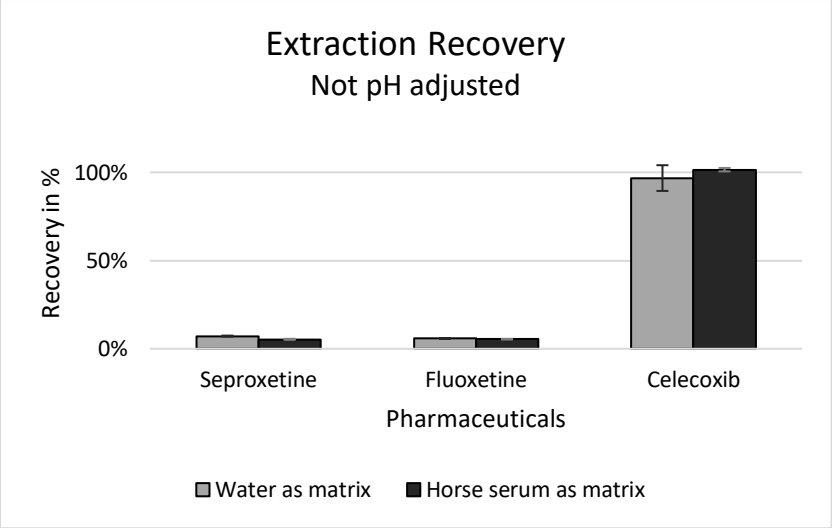


Figure 6. Extraction recovery of seproxetine, fluoxetine and celecoxib without pH adjustment.

Table 1. Ionization suppression of the pharmaceuticals in water and horse serum when no pH adjustment was done.

Ionization suppression	**Water as matrix**	**Horse serum as matrix**
Seproxetine	1%	1%
Fluoxetine	3%	5%
Celecoxib	25%	28%

4.2.2 Alkaline ion-pair extraction (pH 10 and 12)

To improve the extraction efficiency of fluoxetine and seproxetine, the pH of the ion pair solution was adjusted. Since the pKa of both fluoxetine and seproxetine is around 9.8 (Drugbank, 2022), a pH of 12 was evaluated to see if this resulted in a higher recovery than when pH 10 was used. The use of pH 10 is a common practice when performing ion pair extraction for EOF analysis (Hansen et al., 2001) and was therefore also tested. The extraction efficiency when using a high pH increased with approximately 85% (figure 7.) for fluoxetine and seproxetine with a high pH compared to low pH. No observable difference between using pH around 10 or above 12 was observed. The results still showed an ionization suppression for celecoxib, at around 30-40%, which was also noticeable when the pH was not adjusted. Now it is however much more noticeable for fluoxetine (≈ 50%) and seproxetine (≈ 25%) when the recovery was improved. The ionization suppression of each pharmaceutical and matrix is presented in tables 2-3. Since there was not an observable difference in recovery of the pharmaceuticals when using pH 10 and 12, and since pH 10 is of common practice, the pH of around 10 was chosen for further experiments.

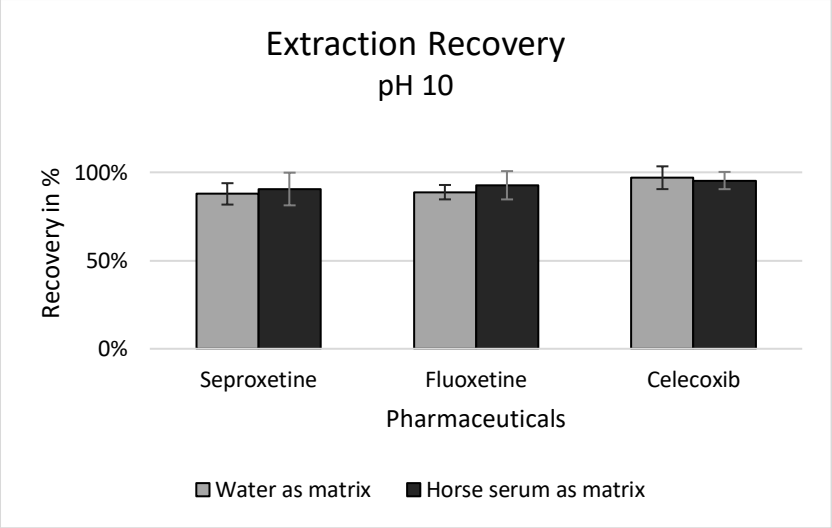


Figure 7. Extraction recovery of seproxetine, fluoxetine and celecoxib with a pH of 10.

Table 2. Ionization suppression of the pharmaceuticals in water and horse serum when a pH of 10 was used.

Ionization suppression	**Water as matrix**	**Horse serum as matrix**
Seproxetine	25%	25%
Fluoxetine	50%	54%
Celecoxib	34%	39%

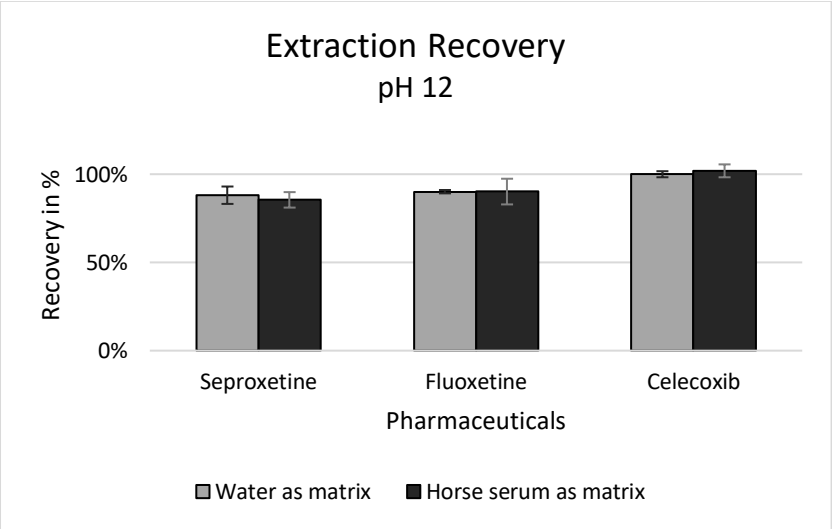


Figure 8. Extraction recovery of seproxetine, fluoxetine and celecoxib with a pH of 12.

Table 3. Ionization suppression of the pharmaceuticals in water and horse serum when a pH of 12 was used.

Ionization suppression	**Water as matrix**	**Horse serum as matrix**
Seproxetine	23%	29%
Fluoxetine	52%	57%
Celecoxib	34%	33%

4.2.3 Recovery of ciprofloxacin

The compound could be detected in the samples where ciprofloxacin were added after the extraction (afterspike samples), but in the samples where the pharmaceutical was spiked before extraction, ciprofloxacin could not be detected. Therefore, it is not included in the graphs above. It might be worth to mention that the chromatographic separation for ciprofloxacin was not ideal, different approaches was made in order to improve it but with no success. However, since the pharmaceutical was seen in the afterspike sample and not in the samples that had gone through extraction it can be stated that it could not be extracted.

4.2.4 The effect of pH on extraction efficiencies on selected fluorinated pharmaceuticals

The pKa of fluoxetine and seproxetine which is approximately 9.8 is most likely the cause of why they are able to be extracted or not in the different uses of pH. In a low pH, the amino group in both fluoxetine and seproxetine would probably be protonated and thus, the chemical will become positively charged. The compounds will then most likely stay in the lower phase i.e., the aqueous phase during the ion pair extraction, and therefore not be extracted. However, if the pH is 10 or higher, the amino group will instead get deprotonated and become negatively charged. Fluoxetine and seproxetine can then form a neutral compound with the ion pair reagent TBA, thus, fluoxetine and seproxetine will be in the upper organic phase which is the phase that is collected. This would explain the large differences in extraction efficiency for these compounds between ion-pair extraction and alkaline ion-pair extraction.

When it comes to Celecoxib, the reason why it can be extracted almost equally well at low and high pH's is due to its lack of base/acid properties. According to the results, whether a low pH (around 3-4), pH 10 or pH 12 is used does not really matter, the compound will still have a very good recovery. Celecoxib is a sulfonamide which is a neutral compound, compared to amines like fluoxetine and seproxetine which properties like bases, and therefore it will not get protonated or deprotonated even if a high or low pH is used. Since it will remain neutral, it will end up in the organic phase and will therefore be extracted no matter what pH is used.

Why ciprofloxacin was not able to be extracted at a low or high pH is most likely due to the ion pair reagent not being able to form a stable ion pair with the pharmaceutical. At a high pH, the carboxyl group would be deprotonated and thus, ciprofloxacin would be negatively charged. Hypothetically it should then end up in the organic phase, the reason why this is not the case, could therefore be because of the ion pair reagent not being sufficient for this type of compound.

4.3 Method evaluation in human sera

Since the extraction only had been performed on horse sera and MilliQ water, the method had to be evaluated for human sera. The extraction was then performed using SRM (SRM 1957) and a pH of 10 to verify if the method could be used on human sera as well. The results are presented figure 9 below and as can be seen the pharmaceuticals showed a bit lower recovery than the extraction tests but still acceptable. Seproxetine and fluoxetine had a recovery of 74% and 83%, respectively, compared to 90% in the extraction test. Celecoxib had a recovery of 92% when in the extraction tests it was around 95-100%. There was also ion suppression as in previous extractions when horse sera and MilliQ water were used which was similar as in the extraction tests (28%-51%).

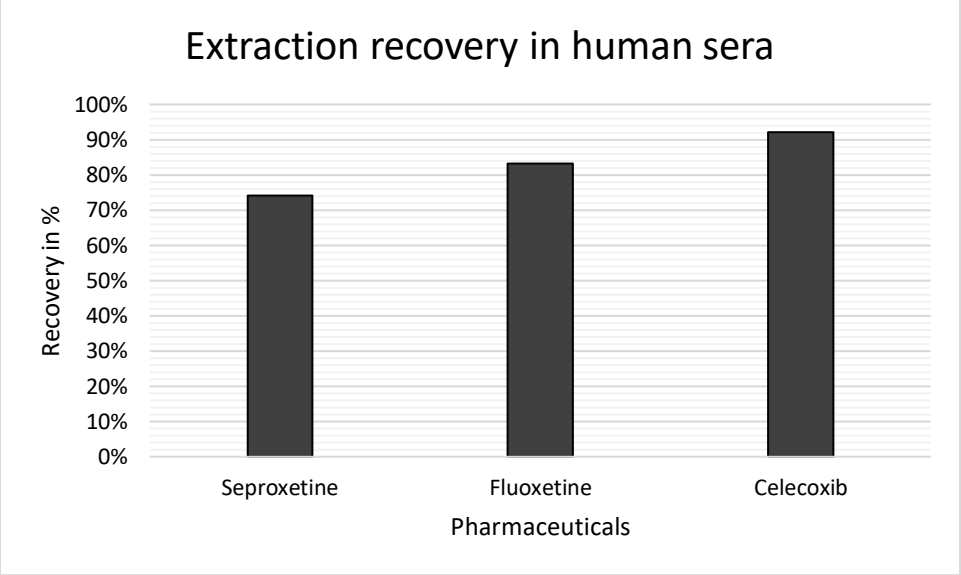


Figure 9. Extraction recovery of seproxetine, fluoxetine and celecoxib in human sera.

4.4 Pharmaceuticals in subjects taken fluoxetine

Blood samples were used to investigate if fluoxetine and its metabolite would be extracted with the ion pair extraction used. Total fluorine content was also measured in these samples to understand if this pharmaceutical would affect the total fluorine content in the blood.

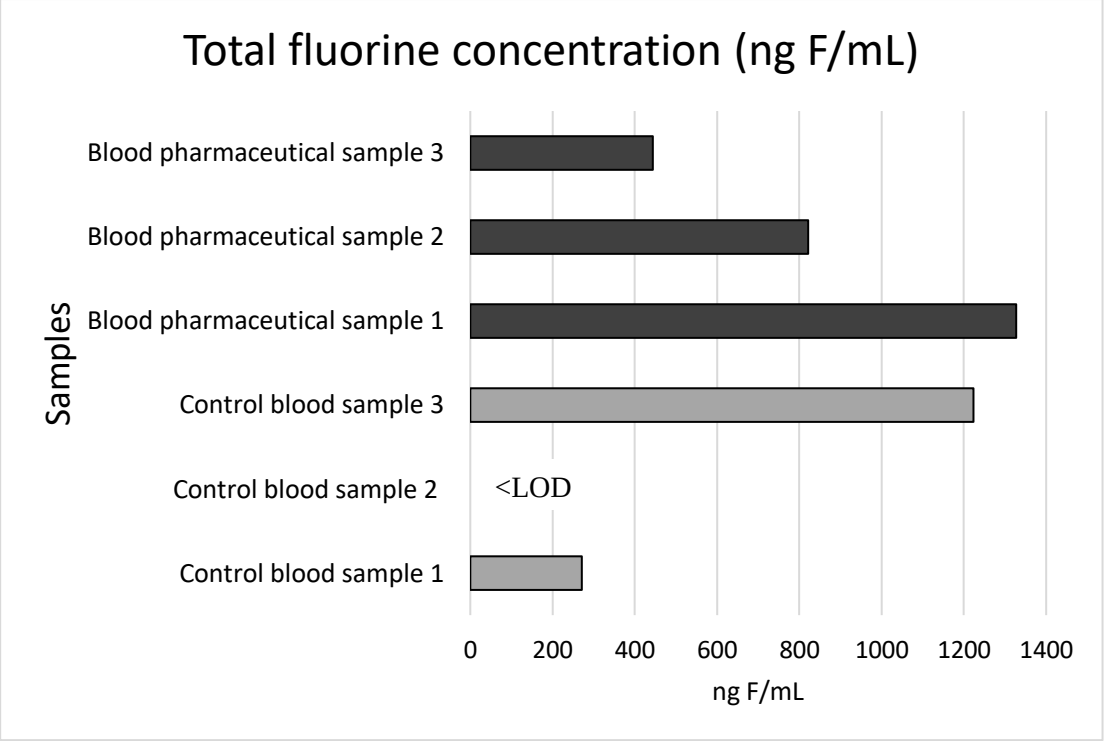
The blood samples (*n*=6) had been already extracted with ion pair extraction without any pH adjustment (pH 3-4) prior to this study, those extracts were analyzed for the four pharmaceuticals (seproxetine, fluoxetine, celecoxib, and ciprofloxacin) in the current study. Even though results from this study showed very low to no recoveries of these compounds in the spike recovery test for the ion-pair extraction with no pH adjustment, it was still worth measuring these compounds in the samples, since if they were present in huge amount in the blood they may have been detected in the extracts. The subjects were known to have taken the pharmaceutical fluoxetine, and since seproxetine is the active metabolite, both could perhaps be expected in the samples. The results showed no detectable levels of these pharmaceuticals in the blood extracts.

Since the results of the current study have shown a good recovery for fluoxetine and seproxetine when using a pH of above 10, it was expected to detect these pharmaceuticals in the extracts where a pH of 10 had been used. However, no detectable level of any of these pharmaceuticals was detected in the blood extracted with alkaline ion-pair. The results from a spiked SRM human sera showed a recovery of 94% for celecoxib, 83% for fluoxetine and 74% for seproxetine which is approximately the same recovery as in the method evaluation for human sera (section 4.3). The good recovery indicated that the extraction was performed in the correct way. The ion suppression was also the same as previous extraction using SRM human sera (28-51%). There could be several explanations to why fluoxetine and seproxetine was not detected, for example metabolism of both the parent compound fluoxetine and the metabolite seproxetine can occur in the serum. The analytes could also go through hydrolysis, oxidation, or isomerization over the time. The presence of lipids and proteins in blood samples also increases the chances of non-covalent binding, sequestration and precipitation which can lead to loss of the analyte (Reed, 2016).

4.4.1 EOF and TF analysis in subjects taken fluoxetine

Since no detectable levels of pharmaceuticals was observed when running the samples on the UPCC, EOF analysis of the blood pharmaceuticals samples was performed. This was done in order to find out if the blood samples where the subjects had taken fluoxetine (blood pharmaceutical samples) might have a higher EOF amount than the control samples, and thus could indicate that the pharmaceuticals had transformed to something else which was not analyzed for. However, there were not any large differences between the control samples and the blood pharmaceutical samples that could indicate this, and the procedural blanks had high EOF contamination which makes it impossible to draw any conclusions from the EOF analysis. The procedural blanks had levels between 50-100 ng F/mL while the blood pharmaceutical samples had <50-117 ng F/mL and the control samples had 67-100 ng F/mL. The high procedural blanks could be due to PFAS contaminations since this have been a problem during the study.

A total fluorine analysis was also performed to see if there could be any differences in the total fluorine amount between the control samples and the blood samples. Since the pharmaceuticals could have been metabolized, they might be present, but in another form that could not be extracted in the current extraction method used. Therefore, the total fluorine analysis was of interest. The results from the TF analysis are presented in graph 5. As can be seen, the fluorine content of control blood sample 2 were below LOD (100 ng F/mL). However, the nitrate from the silver nitrate used to remove chloride is probably suppressing the signals from fluoride, but since it is still detected in the other samples it could be that the amount is very small and therefore not detectable due to the suppression. Except for the control blood sample 3, the blood pharmaceutical samples 1-3, which is the ones where the subjects had taken fluoxetine, seem to have a higher TF concentration. There is unfortunately very little knowledge about the specific blood bsamples, for example how much pharmaceutical the subject has consumed and if there could be anything else that could contribute to the fluoride concentration. What also must be considered is that since the total fluorine analysis has not gone through any extraction, there could be presence of inorganic fluorine. The background level of inorganic fluor could vary between continents as well as different periods in time of collection which makes the results difficult to compare with other studies. However, the measured TF values are in the same magnitude as other studies (Yeung et al., 2008, Miyake et al., 2007). Although vague, the results could show some indication that the blood samples have a higher amount of total fluorine, at least higher than two of the control samples. This can indicate that the pharmaceuticals in fact might be present in the blood samples but not extractable with the ion pair extraction. Why it cannot be extracted is something that needs to be further studied. Since the results show a very large variation between the samples, and since one of the control samples (control blood sample 3) has a quite high TF concentration, no conclusion can be made that there in fact is a difference in the TF concentration between the subjects that had taken fluoxetine and the ones that had not. A Student t-test was performed in order to investigate of there was any significant difference between these two groups, which resulted in a value of 0.46, thus, there is not any significant difference.



Graph 5. Total fluorine concentration in blood samples (*n*= 6).

5. Future aspects

According to the results, celecoxib, fluoxetine and seproxetine did get extracted in alkaline ion pair extraction when the pharmaceuticals were spiked prior the extraction. If fluoxetine and/or seproxetine is present in the blood samples can't be stated for sure, but the TF analysis shows an indication of a higher TF amount than in the control samples if control sample 3 is not considered. If this is the case, further work is needed to determine the fate of the pharmaceuticals in the blood samples. Oxidation and degradation experiments could be conducted to find information about the transformation products. Hydrolysis could also be performed to release the drugs from any potential conjugate that could have been formed in the blood samples.

The information about the blood samples in this study are little, for example it is not known for how long the subjects have been consuming fluoxetine or how much. Therefore, further extraction, using pH 10 should be conducted with samples where these things are known to be able to evaluate hypothetic concentration of the pharmaceutical.

The results also show that celecoxib is extracted no matter a low or high pH is used. A future aspect is to do the extraction on blood samples where the subject had consumed celecoxib to see if it can be extracted then as well. Another future aspect could also be to try another ion pair reagent for extraction of ciprofloxacin.

Since ionization suppression was observed for the four pharmaceuticals, matrix-match calibration curve or standard addition are needed to quantify these chemicals in sera samples.

6. Conclusion

The extraction tests showed that fluoxetine and seproxetine have a good recovery in ion pair extraction when using a pH of 10 or above, and when using a low pH of 3-4 they will barely be extracted at all. The ion pair extraction performed on the blood samples where subjects had taken fluoxetine did not show any detectable presence of fluoxetine or its metabolite seproxetine, even though a pH of 10 was used. However, this could be due to the fact that the analyte may not be present in the samples anymore or that it has been metabolized into something that wasn't extracted, the TF analysis does in fact give some indication that this might be the case, and that the pharmaceutical is present. However, the large variations between the samples make it hard to draw any conclusion. Despite this, if any blood samples are to be analyzed for EOF as a proxy for amount of PFAS using ion pair extraction, and it is known that the subject has been consuming fluoxetine or any other fluorinated pharmaceutical with similar structure, it should be considered to use a low pH to make sure the pharmaceutical won't contribute to the EOF amount. The question if the pharmaceuticals (fluoxetine and seproxetine) is present in another form or even at all in the blood samples remains.

Although celecoxib is extracted well in the extraction tests, no conclusions can be drawn to if it may contribute to the EOF amount in human blood. It can be said that it hypothetically could contribute, but, since no extraction on real blood samples that contained celecoxib was performed it cannot be stated for sure.

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Appendix

Table 1. Parameters for individual pharmaceuticals.

Analyte	Precursor/ product ions quantification (m/z)	Cone (V)	Col (eV)	Precursor/ product ions qualification (m/z)	Cone (V)	Col (eV)
Seproxetine	296.15/134.00	20	6	296.15/30.00	20	10
Fluxoetine	310.15/44.00	20	10	310.15/148.00	20	8
Celecoxib	382.10/362.20	20	35	382.10/282.20	20	29
Ciprofloxacin	332.10/314.00	25	19	332.10/288.00	25	17

Table 2. UPCC method for pharmaceuticals

t (min)	Mobile phase A (CO_2) (%)	Mobile phase B (%)
0.00	80	20
3.00	65	35
3.50	55	45
4.50	55	45
4.51	80	20
5.50	80	20

Table 3. TQSμ-MS/MS Parameters.

Ionization source:	ESI positive mode
Source temperature:	150 °C
Desolvation temperature:	450 °C
Desolvation gas flow:	1000 L/h
Cone gas flow:	150 L/h
Capillary:	4.00 kV

Table 4. Parameters for individual PFAS.

Analyte	Precursor/ product ions quantification (m/z)	Cone (V)	Col (eV)	Precursor/ product ions qualification (m/z)	Cone (V)	Col (eV)	Corresponding Internal Standard
PFBS	298.90/98.90	20	26	298.90/79.96	20	26	$^{13}C_3$ PFBS
PFPeS	348.90/98.96	20	26	348.90/79.96	20	30	$^{18}O_2$ PFHxS
L-PFHxS	398.90/98.90	20	30	398.90/79.96	20	34	$^{18}O_2$ PFHxS
PFHpS	448.97/98.90	20	30	448.97/79.96	20	35	$^{13}C_4$ PFOS
L-PFOS	498.97/98.96	20	38	498.97/79.96 498.97/169.03	20	44 34	$^{13}C_4$ PFOS
PFNS	548.90/98.96	20	38	548.90/79.96	20	44	$^{13}C_4$ PFOS
PFDS	598.97/98.90	20	42	598.97/79.96	20	58	$^{13}C_4$ PFOS
PFDoDS	698.97/98.90	20	40	698.97/79.96	20	45	$^{13}C_4$ PFOS
4:2 FTSA	327.00/307.00	20	20	327.00/81.00	20	28	$^{13}C_2$ 6:2 FTSA
6:2 FTSA	427.00/407.00	20	20	427.00/81.00	20	28	$^{13}C_2$ 6:2 FTSA
8:2 FTSA	527.00/507.00	20	20	527.00/80.00	20	28	$^{13}C_2$ 8:2 FTSA

PFBA	212.97/169.00	20	11	-	-	-	$^{13}C_4$ PFBA
PFPeA	262.97/219.00	20	8	-	-	-	$^{13}C_3$ PFPeA
PFHxA	312.97/269.00	20	9	312.97/118.95	20	26	$^{13}C_2$ PFHxA
PFHpA	362.97/319.00	20	10	362.97/168.97	20	16	$^{13}C_2$ PFHxA
L-PFOA	412.97/369.00	20	10	412.97/168.97	20	18	$^{13}C_4$ PFOA
PFNA	462.99/419.00	20	12	462.99/219.00	20	18	$^{13}C_5$ PFNA
PFDA	512.97/469.00	20	11	512.97/219.00	20	18	$^{13}C_2$ PFDA
PFUnDA	562.97/519.00	20	12	562.97/268.99	20	18	$^{13}C_2$ PFUnDA
PFDoDA	612.97/569.00	34	14	612.97/168.96	40	22	$^{13}C_2$ PFDoDA
PFTrDA	662.90/619.00	20	14	662.90/168.96	20	26	$^{13}C_2$ PFDoDA
PFTDA	712.90/669.00	20	14	712.90/168.97	20	28	$^{13}C_2$ PFTDA
PFHxDA	812.90/769.00	30	15	812.90/168.96	42	32	$^{13}C_2$ PFHxDA
PFOcDA	912.90/869.00	36	15	912.90/168.96	36	36	$^{13}C_2$ PFHxDA

Table 5. UPLC method for target PFAS.

t (min)	Mobile phase A (%)	Mobile phase B (%)
0.00	99	1
0.57	99	1
13.00	0	100
14.00	0	100
14.20	99	1
17.00	99	1

Table 6. TQS-MS/MS Parameters.

Ionization source:	ESI negative mode
Source temperature:	150 °C
Desolvation temperature:	400 °C
Desolvation gas flow:	800 L/h
Cone gas flow:	150 L/h
Capillary:	0.70 kV

Table 7. LOD values for individual PFAS and individual extraction tests.

	No pH adjusment extraction test		Alkaline extraction test (pH 10)		Alkaline extraction test (pH 12)	
LOD	Water as matrix	Horse serum as matrix	Water as matrix	Horse serum as matrix	Water as matrix	Horse serum as matrix
PFBA (ng/mL)	0,100	0,100	0,100	0,100	0,010	0,165
PFPeA (ng/mL)	0,082	0,067	0,067	0,089	0,074	0,068
PFBS (ng/mL)	0,090	0,090	0,090	0,090	0,090	0,090
PFHxA (ng/mL)	0,050	0,050	0,050	0,050	0,050	0,050
PFHpA (ng/mL)	0,100	0,100	0,100	0,100	0,100	0,100
PFPeS (ng/mL)	0,050	0,050	0,050	0,050	0,050	0,050
PFHxS (ng/mL)	0,078	0,112	0,087	0,088	0,061	0,084
PFHpS (ng/mL)	0,050	0,050	0,050	0,050	0,050	0,050
PFOA (ng/mL)	0,100	0,100	0,100	0,100	0,100	0,100
PFNA (ng/mL)	0,050	0,050	0,050	0,050	0,050	0,050
PFOS99 (ng/mL)	0,050	0,182	0,050	0,136	0,146	0,283
PFDA (ng/mL)	0,100	0,100	0,100	0,100	0,100	0,100
PFUnDA (ng/mL)	0,020	0,036	0,020	0,051	0,020	0,039
PFNS (ng/mL)	0,040	0,040	0,040	0,040	0,040	0,040

PFDS (ng/mL)	0,020	0,020	0,020	0,020	0,020	0,020
PFDoDA (ng/mL)	0,050	0,050	0,050	0,050	0,050	0,050
PFTrDA (ng/mL)	0,100	0,100	0,100	0,100	0,100	0,100
PFDoDS (ng/mL)	0,090	0,090	0,090	0,090	0,090	0,090
PFTDA (ng/mL)	0,027	0,024	0,020	0,028	0,020	0,020
PFHxDA (ng/mL)	0,193	0,187	0,188	0,210	0,215	0,200
PFOcDA (ng/mL)	0,020	0,026	0,020	0,040	0,020	0,020
4_2_FTSA (ng/mL)	0,050	0,050	0,050	0,050	0,050	0,050
6_2_FTSA (ng/mL)	0,090	0,090	0,090	0,090	0,313	0,090
8_2_FTSA (ng/mL)	0,100	0,100	0,100	0,100	0,100	0,100
PFOSA (ng/mL)	0,100	0,100	0,100	0,100	0,100	0,100

Table 8. Native PFAS in the CS mixture.

Perfluorobutanoic acid (PFBA)	Perfluorononanoic acid (PFNA)	Perfluorotetradecanoic acid (PFTeDA)	Perfluorooctane sulfonic acid (PFOS)	8:2 Fluorotelomer sulfonic acid (8:2 FTSA)
Perfluoropentanoic acid (PFPeA)	Perfluorodecanoic Acid (PFDA)	Perfluorohexadecanoic acid (PFHxDA)	Perfluorodecane sulfonic acid (PFDS)	Perfluoroheptane sulfonic acid (PFHpS)
Perfluorohexanoic acid (PFHxA)	Perfluoroundecanoic acid (PFUnDA)	Perfluorooctadecanoic acid (PFODA)	Perfluorooctane sulfonamide (PFOSA)	Perfluorododecane sulfonic acid (PFDOS)
Perfluoroheptanoic acid (PFHpA)	Perfluorododecanoic acid (PFDoDA)	Perfluorobutane sulfonic acid (PFBS)	4:2 Fluorotelomer sulfonic acid (4:2 FTSA)	Perfluorononane sulfonic acid (PFNS)
Perfluorooctanoic acid (PFOA)	Perfluorotridecanoic acid (PFTrDA)	Perfluorohexane sulfonic acid (PFHxS)	6:2 Fluorotelomer sulfonic acid (6:2 FTSA)	Perfluoropentane sulfonic acid (PFPeS)

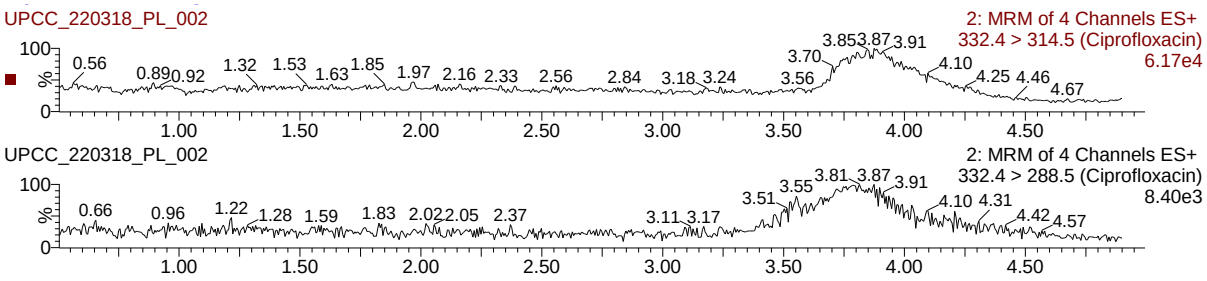
Table 9. Mass labeled PFAS in the IS mixture.

Perfluorooctane sulfonamide (PFOSA)	6:2 Fluorotelomer sulfonic acid (6:2 FTSA)	Perfluoropentanoic acid (PFPeA)	Perfluorohexanoic acid (PFHxA)	Perfluorodecanoic Acid (PFDA)
Perfluorohexadecanoic acid (PFHxDA)	8:2 Fluorotelomer sulfonic acid (8:2 FTSA)	Perfluoroheptanoic acid (PFHpA)	Perfluorooctanoic acid (PFOA)	Perfluoroundecanoic acid (PFUnDA)
Perfluorotetradecanoic acid (PFTeDA)	Perfluorobutane sulfonic acid (PFBS)	Perfluorobutanoic acid (PFBA)	Perfluorononanoic acid (PFNA)	Perfluorododecanoic acid (PFDoDA)
Perfluorohexane sulfonic acid (PFHxS)	Perfluorooctane sulfonic acid (PFOS)			

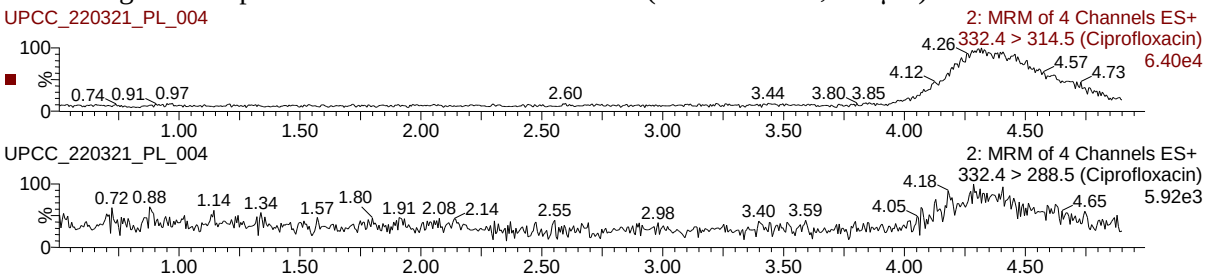
Table 10. Mass labeled PFAS in the RS mixture.

Perfluorobutanoic acid (PFBA)	Perfluorooctane sulfonic acid (PFOS)	Perfluorononanoic acid (PFNA)	Perfluoroundecanoic acid (PFUnDA)	Perfluorohexanoic acid (PFHxA)
Perfluorohexane sulfonic acid (PFHxS)	Perfluorooctanoic acid (PFOA)	Perfluorodecanoic Acid (PFDA)	4:2 Fluorotelomer sulfonic acid (4:2 FTSA)	Perfluoropentanoic acid (PFPeA)

Chromatogram of ciprofloxacin with the Torus DIOL analytical column (3.0 × 100 mm, 1.7 µm).



Chromatogram of ciprofloxacin with the BEH column (3.0 × 100 mm, 1.7 µm) .



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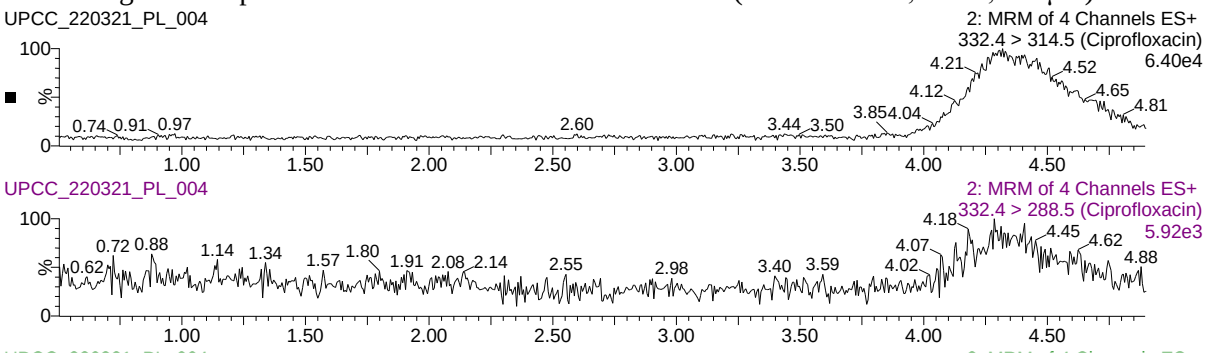

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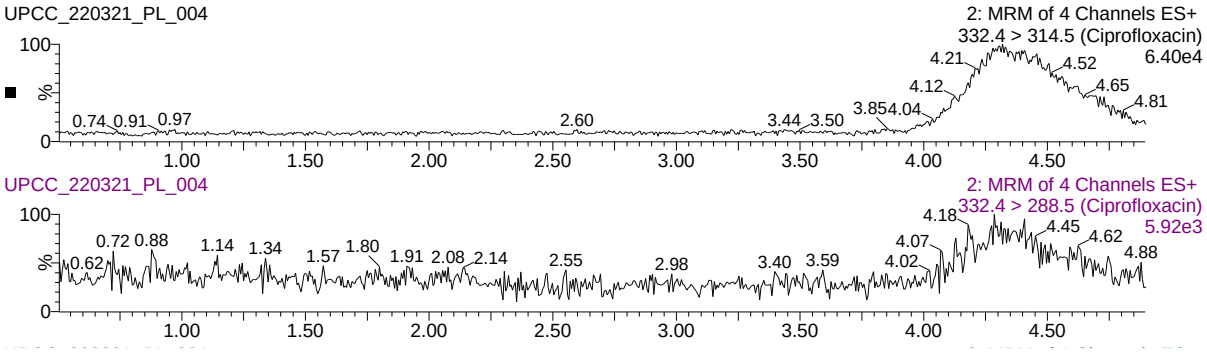
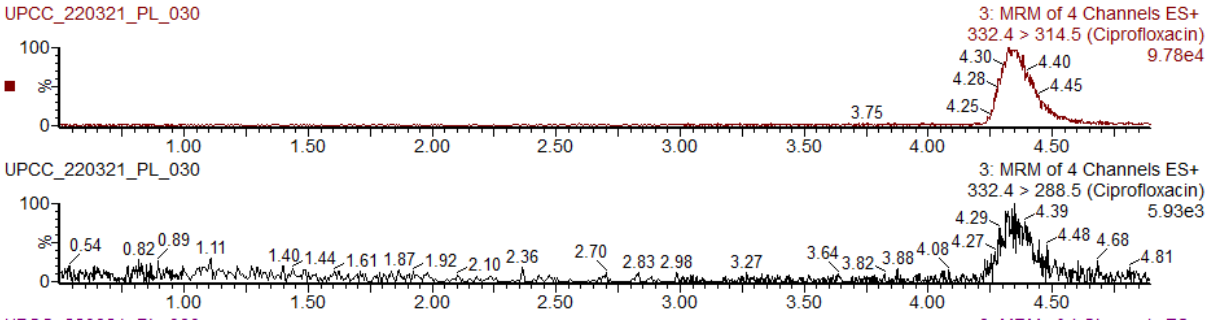
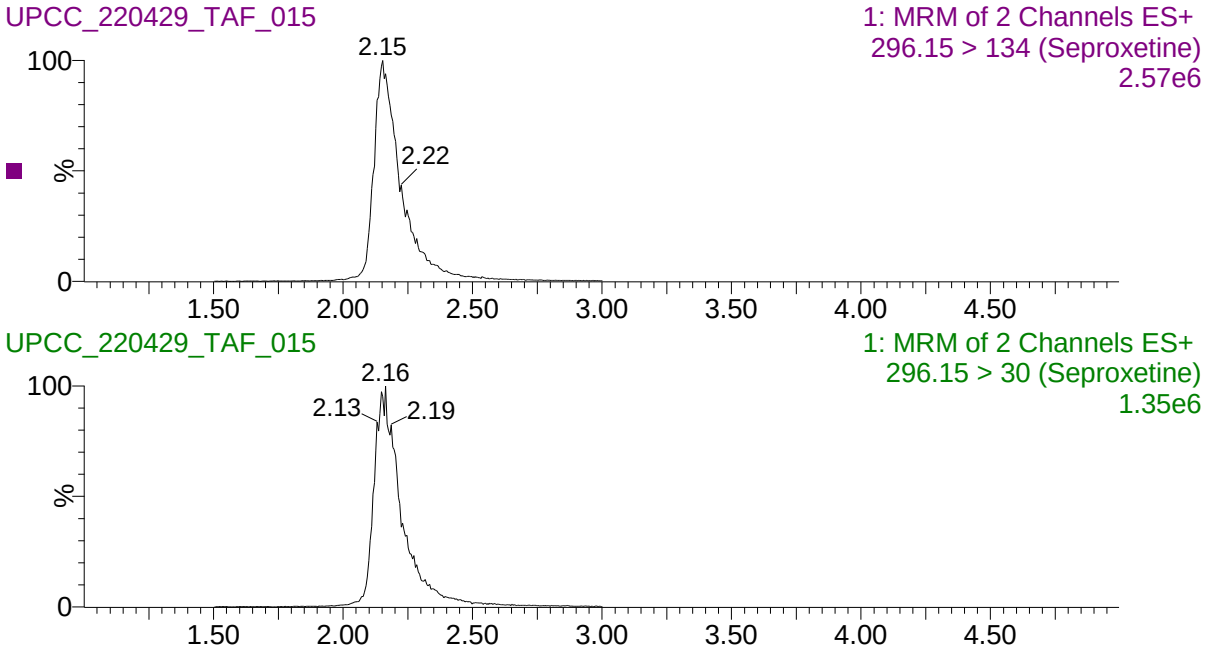


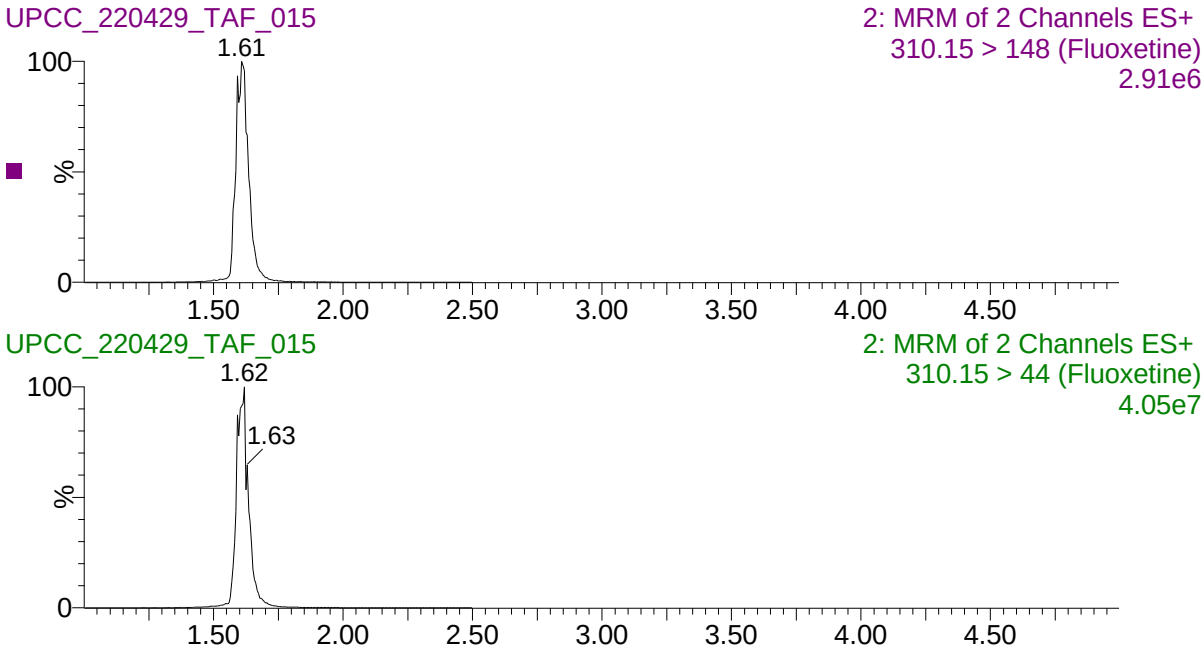
Chromatogram of ciprofloxacin with the DIOL Torus DIOL analytical column (3.0 × 100 mm, 1.7 µm) and 0.5% NH_4OH in MeOH.



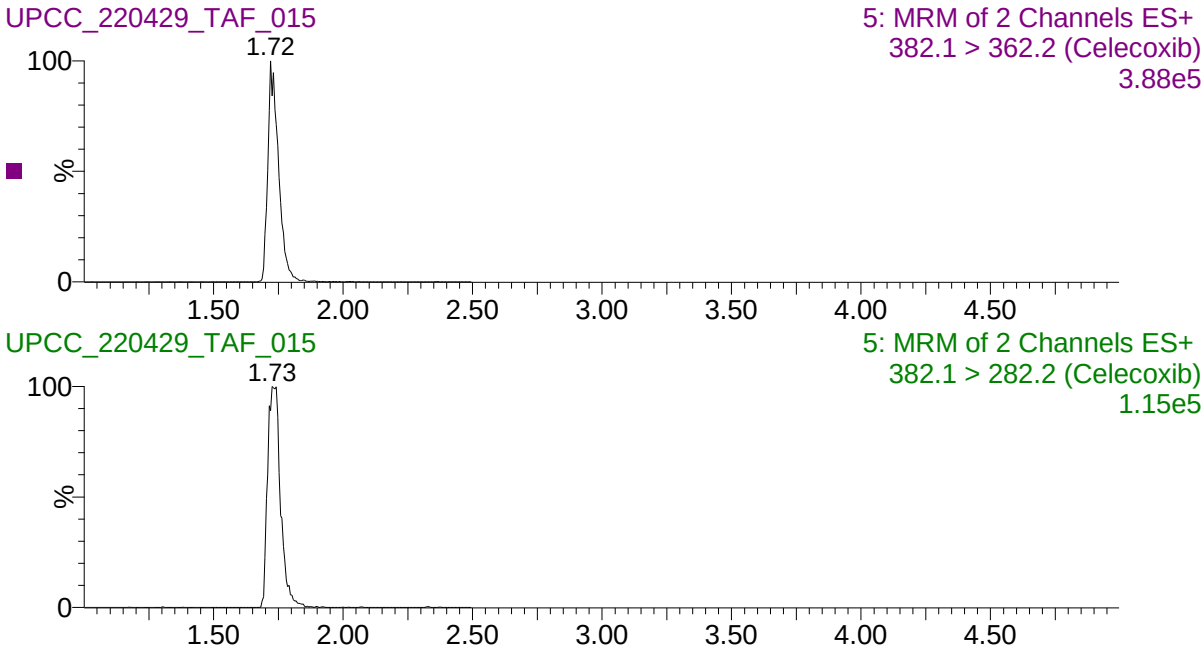
Chromatogram of seproxetine with the DIOL Torus DIOL analytical column (3.0 × 100 mm, 1.7 µm) and 0.05% NH_4OH in MeOH.



Chromatogram of fluoxetine the DIOL Torus DIOL analytical column (3.0 × 100 mm, 1.7 µm) and 0.05% NH_4OH in MeOH.



Chromatogram of celecoxib the DIOL Torus DIOL analytical column (3.0 × 100 mm, 1.7 µm) and 0.05% NH_4OH in MeOH.



Chromatogram of ciprofloxacin the DIOL Torus DIOL analytical column (3.0 × 100 mm, 1.7 μm) and 0.05% NH_4OH in MeOH.